

# Convergence of MAP kinase pathways on the ternary complex factor Sap-1a

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**The serum response element (SRE), which is pivotal for transcriptional up-regulation of the *c-fos* proto-oncogene, is constitutively occupied by a protein complex comprising the serum response factor and a ternary complex factor (TCF). Phosphorylation of the TCFs Elk-1 and Sap-1a by the ERK and JNK subclasses of MAP kinases triggers *c-fos* transcription. We demonstrate here that Elk-1 is barely activated by a third subclass of MAP kinases (p38), most likely because the critical residues Ser383 and Ser389 are poorly phosphorylated by p38 MAP kinase. In contrast, the TCF Sap-1a is efficiently phosphorylated by p38 MAP kinase *in vitro* and *in vivo* on the homologous residues Ser381 and Ser387. Mutation of these sites to alanine severely reduces *c-fos* SRE-dependent transcription mediated by Sap-1a and p38 MAP kinase. Thus, Sap-1a may be an important target for mitogens, stress and apoptotic signals to elicit a nuclear response. However, signaling from p38 MAP kinase to Sap-1a or from Sap-1a to the basal transcription machinery does not occur in all cell types nor at promoters other than the *c-fos* SRE, which may ensure the specificity of signaling.**

**Keywords:** *c-fos*/ETS protein/phosphorylation/signal transduction/transcription

## Introduction

The *c-fos* proto-oncogene is a paradigmatic immediate early gene which is activated within minutes by a variety of extracellular stimuli, including serum, growth factors, phorbol esters, cytokines, neurotransmitters, Ca<sup>2+</sup>, UV light and redox agents. Most of these signals target the *c-fos* promoter at three distinct sites, the *Sis*-inducible element (SIE), the serum response element (SRE) or the cAMP response element (CRE) (Janknecht *et al.*, 1995a). However, analysis of transgenic mice has revealed that the SRE is of pivotal importance, since its presence is even required for signals entering the *c-fos* promoter via the SIE or CRE to elicit a maximal increase in *c-fos* mRNA production (Robertson *et al.*, 1995).

Genomic footprinting analyses showed that the *c-fos* SRE is constitutively occupied by a protein complex consisting of the serum response factor (SRF) and a ternary complex factor (TCF) (Herrera *et al.*, 1989). Up to now, three TCFs have been identified (Elk-1, Sap-1a and Sap-2/Erp/Net), which are members of the ETS

transcription factor family (Janknecht *et al.*, 1995a). One signature motif of the TCF proteins is a 20 amino acid sequence which mediates protein–protein interaction with the SRF protein (Shore and Sharrocks, 1994). This is essential for stable binding of a TCF to the *c-fos* SRE, since the SRE contains only a very low affinity binding site for TCFs. Although the SRF protein is phosphorylated upon growth factor stimulation (Rivera *et al.*, 1993), no evidence has been presented that this phosphorylation affects *c-fos* transcription. On the contrary, phosphorylation of TCFs by the ERK subclass of MAP kinases (MAPKs) is critical for activation of the *c-fos* gene (Janknecht *et al.*, 1993, 1995b; Marais *et al.*, 1993; Giovane *et al.*, 1994; Price *et al.*, 1995).

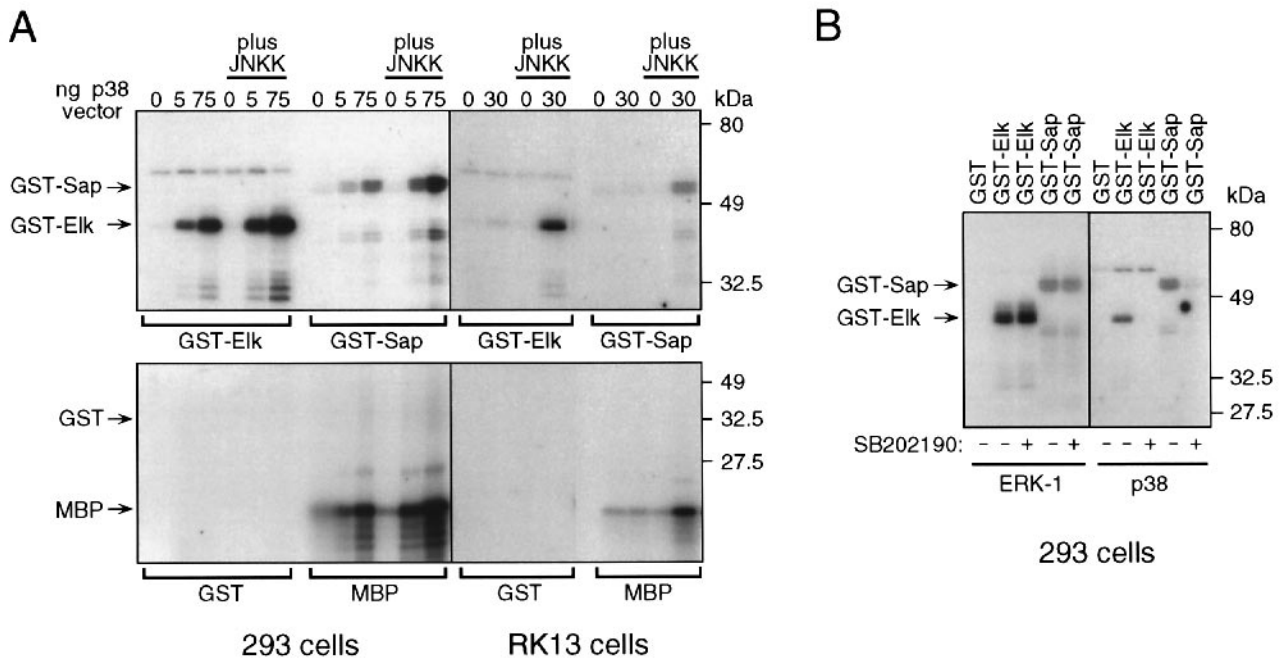
While ERKs are mainly activated by mitogenic signals, the JNK subclass of MAPKs is regarded as a sensor for stress signals (Cahill *et al.*, 1996; Pelech, 1996). Recently, it has been shown that the TCF Elk-1 is also phosphorylated and thus activated by JNKs (Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995). A third major subclass of mammalian MAPKs is represented by p38 and the recently cloned p38 $\beta$  (Jiang *et al.*, 1996; Pelech, 1996). These kinases are, often in conjunction with JNKs, activated under conditions of cellular stress elicited by UV light, hyperosmolarity, arsenite or transforming growth factor- $\beta$  (TGF- $\beta$ ). Although MAPKAP kinase-2 and the transcription factor CHOP have been identified as proteins phosphorylated by p38 MAPK (Rouse *et al.*, 1994; Wang and Ron, 1996), little more is known about the intracellular targets of p38 MAPK.

Here we demonstrate that p38 MAPK phosphorylates the TCFs Sap-1a and Elk-1. However, while Sap-1a is activated through this phosphorylation and thus gains the ability to stimulate *c-fos* SRE-dependent transcription in 293 cells, Elk-1 is barely activated, since important phosphorylation sites are not efficiently recognized by p38 MAPK. Yet p38 MAPK activity does not lead to significant Sap-1a-mediated *c-fos* SRE-stimulation in RK13 cells nor to activation of a promoter regulated by Sap-1a autonomously of the SRF protein in 293 cells. This suggests that signaling via p38 MAPK and Sap-1a is restricted and thus rather specific.

## Results

### *p38 MAPK phosphorylates Sap-1a and Elk-1 in vitro*

p38 MAPK was transiently expressed in human embryonal kidney 293 cells and immunoprecipitated via its flag tag with the M2 antibody. The immunoprecipitates were incubated with purified GST fusion proteins containing the C-terminal activation domain of either Sap-1a or Elk-1 (amino acids 268–431 and 307–428 respectively) in the



**Fig. 1.** *In vitro* phosphorylation of GST-Elk or GST-Sap by p38 MAPK. (A) p38 MAPK was produced in transiently transfected 293 or RK13 cells. Where indicated, JNKK was co-transfected. After immunoprecipitation, p38 MAPK was incubated with comparable amounts of GST fusion proteins or MBP in the presence of [ $\gamma$ - $^{32}$ P]ATP. Shown are autoradiographs after SDS-PAGE. (B) Inhibition of *in vitro* phosphorylation by p38 MAPK, but not by ERK-1, in the presence of 2  $\mu$ M SB202190.

presence of [ $\gamma$ - $^{32}$ P]ATP. Similarly to the classic MAPK substrate myelin basic protein (MBP), both GST-Sap and GST-Elk were phosphorylated by p38 MAPK in a dose-dependent fashion, while the GST moiety alone was not phosphorylated (Figure 1A, left panels). Furthermore, co-expression of the human JNK kinase (JNKK; also known as MKK4 or SEK1), which is a direct upstream activator of both p38 MAPK and JNKs (Lin *et al.*, 1995), potentiated the ability of p38 MAPK to phosphorylate MBP, GST-Elk and GST-Sap, but had no effect on its own (Figure 1A, left panels).

In contrast to 293 cells, transient expression of p38 MAPK in rabbit kidney epithelial-like RK13 cells did not lead to a significant activation of p38 MAPK (Figure 1A, right panels), which holds true even for 100-fold higher amounts of p38 MAPK expression vector employed in the transfection experiments (data not shown). Rather, JNKK had to be co-expressed to detect phosphorylation of GST-Elk, GST-Sap and MBP by p38 MAPK immunoprecipitates *in vitro*. These data imply that 293 cells have an endogenous activator of the p38 MAPK pathway or alternatively lack a negative regulator, which may be due to the transformed nature of 293 cells in contrast to RK13 cells.

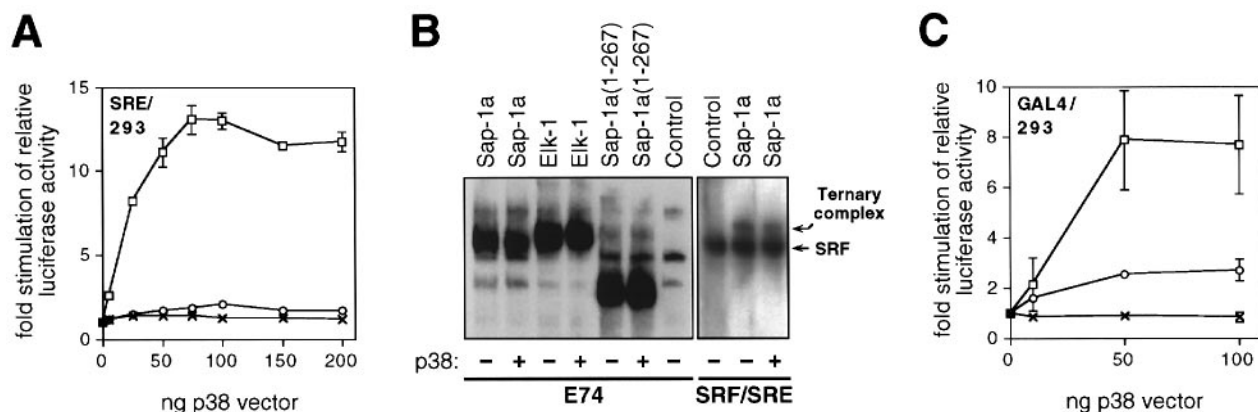
p38 MAPK activity can be blocked specifically by the compound SB202190 (Lee *et al.*, 1994). Consistently, the *in vitro* phosphorylation of GST-Elk and GST-Sap by immunoprecipitated p38 MAPK was abolished in the presence of the inhibitor SB202190, whereas the ability of the MAPK ERK-1 to phosphorylate these two TCFs was unaffected (Figure 1B).

#### p38 MAPK activates Sap-1a-mediated transcription

Phosphorylation of a transcription factor does not necessarily have an impact on its function. Thus, we tested

whether p38 MAPK phosphorylation of TCFs led to transcriptional activation of the *c-fos* SRE in 293 cells. To that end, a *c-fos* SRE-driven luciferase reporter construct was co-transfected with expression vectors for a TCF and p38 MAPK. No exogenous SRF was expressed, since the endogenous level of SRF is sufficiently high; further, more expression of exogenous SRF has been reported to lead to squelching (Hill *et al.*, 1993; Ernst *et al.*, 1995). Luciferase reporter gene activity in the presence of Sap-1a was stimulated >12-fold by p38 MAPK in a dose-dependent manner in 293 cells (Figure 2A). Surprisingly, Elk-1 was at most 2-fold activated and the control protein Sap-1a(1-267), which lacks the C-terminal transactivation domain (Janknecht *et al.*, 1995b), was, as expected, unresponsive to p38 MAPK. These effects were not due to different levels of TCF expression, since a similar degree of complex formation with the E74 site, to which Elk-1 and Sap-1a bind with comparable affinities in the absence of SRF (Janknecht *et al.*, 1995b), was observed (Figure 2B). Furthermore, the increase in Sap-1a-mediated *c-fos* SRE activation did not correlate with a change in DNA binding affinity, since neither binding to the E74 site nor ternary complex formation with the SRF protein at the *c-fos* SRE was affected by p38 MAPK expression (Figure 2B).

Next, we investigated the behavior of the C-terminal transactivation domains of Sap-1a and Elk-1 in response to p38 MAPK. To that end, the C-terminal activation domains of the two TCFs were fused to the DNA binding domain of the yeast protein GAL4 and the activity of the fusion proteins was assessed with a luciferase reporter gene driven by two GAL4 binding sites in 293 cells (Figure 2C). The GAL4-Sap fusion protein was activated by p38 MAPK expression in a dose-dependent manner. However, the maximal degree of activation was smaller



**Fig. 2.** Transactivation mediated by Sap-1a and p38 MAPK *in vivo*. (A) Dose-dependent stimulation of *c-fos* SRE-driven luciferase reporter gene activity by p38 MAPK and the TCFs Sap-1a (rectangles), Elk-1 (circles) or Sap-1a(1-267) (crosses) in 293 cells. (B) Gel retardation assays with  $^{32}$ P-labeled E74 oligonucleotide or  $^{32}$ P-labeled *c-fos* SRE plus the SRF protein and extracts of 293 cells that were transiently transfected with TCF and p38 MAPK expression vectors as indicated. Control denotes an extract from mock-transfected 293 cells. (C) Effect of p38 MAPK on a GAL4 binding site-driven luciferase reporter in the presence of the GAL4 moiety (crosses), GAL4-Elk (circles) or GAL4-Sap (rectangles) in 293 cells.

than that observed with the full-length Sap-1a molecule in Figure 2A, which contrasts with what we have observed upon ERK-1 activation, where the respective GAL4-Sap fusion protein was ~10-fold more active than the full-length protein (Janknecht *et al.*, 1995b; R.Janknecht and T.Hunter, unpublished data). This suggests that p38 MAPK activation of GAL4-Sap is very inefficient, rendering it unsuitable as a tool to study p38 MAPK activation of Sap-1a. Therefore, the analysis of full-length proteins is indispensable to unravel the impact of a protein kinase on the function of TCFs. As reported by Raingeaud *et al.* (1996), we also observed a small activation of the GAL4-Elk fusion protein by p38 MAPK (Figure 2C), but this activation was substantially less than that obtained with GAL4-Sap, in accordance with the fact that Sap-1a was functionally more susceptible to activation by p38 MAPK than Elk-1.

p38 MAPK-induced transactivation by Sap-1a could be suppressed by the inhibitor SB202190 *in vivo*, whereas stimulation by ERK-1 in the presence of constitutively activated Raf-1 (BXB), which is a MAPK kinase kinase upstream of ERK-1, was not blocked by SB202190 (Figure 3A). Transactivation mediated by Sap-1a upon p38 MAPK expression in 293 cells was enhanced by JNKK and was comparable with transactivation upon ERK-1/BXB stimulation (Figure 3A). Also, TAK1, which is a MAPK kinase kinase acting upstream of p38 MAPK (Yamaguchi *et al.*, 1995; Moriguchi *et al.*, 1996), synergized with p38 MAPK to stimulate Sap-1a- but not Elk-1-driven transcription (Figure 3B). Note that we employed only 25 ng p38 MAPK expression plasmid, since cooperation can best be observed at nearly threshold levels for each component; nevertheless, we also observed cooperation between TAK1 and p38 MAPK when 75 ng p38 MAPK expression plasmid were used for the transfection studies, although the degree of cooperation was smaller (data not shown). Collectively, these results indicate that phosphorylation of Sap-1a, but not Elk-1, by p38 MAPK leads to efficient activation of the *c-fos* SRE in 293 cells.

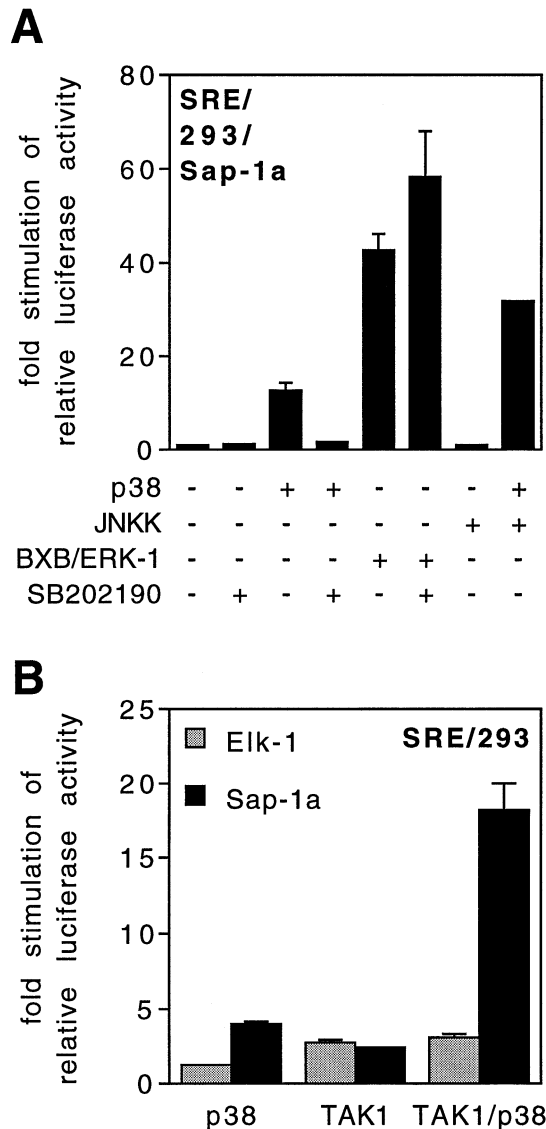
We then performed equivalent transactivation studies in RK13 cells. Consistent with the *in vitro* kinase assays described in Figure 1A, transfection of 30 ng p38 MAPK vector (Figure 4) or up to 100-fold higher amounts of p38

MAPK vector (data not shown) into RK13 cells did not lead to any activation of *c-fos* SRE-dependent transcription mediated by Sap-1a (Figure 4). We expected to observe transactivation upon joint expression of JNKK and p38 MAPK leading to p38 MAPK activation as shown in Figure 1A, but surprisingly Sap-1a was only marginally activated under these conditions and thus behaved similarly to Elk-1 and Sap-1a(1-267). Note that both Elk-1 and Sap-1a can be strongly activated in RK13 cells, as we have shown previously upon ERK stimulation (Janknecht *et al.*, 1995b). Thus, p38 MAPK activation does not necessarily lead to Sap-1a-mediated *c-fos* SRE stimulation in all cell types.

Since TCFs can act not only in a complex with the SRF protein but also independently of SRF at various ETS binding sites (Janknecht *et al.*, 1995b), we tested the activation of an E74 site-driven luciferase reporter gene in 293 cells. In contrast to the *c-fos* SRE, Sap-1a resembled Elk-1 in that it only stimulated transcription via the E74 site upon p38 MAPK expression by 2- to 3-fold (Figure 5). This is not due to the fact that Sap-1a binds less tightly to the E74 site than to the *c-fos* SRE in the presence of SRF, since binding affinities are comparable (Janknecht *et al.*, 1995b). Also, the ability of Sap-1a to be activated upon ERK stimulation was greatly reduced relative to transcription via the *c-fos* SRE (compare Figures 5 and 3A). This suggests that Sap-1a is much more potent in a ternary complex with the SRF protein at the *c-fos* SRE than it is without SRF at the E74 site in 293 cells.

#### Identification of major p38 MAPK phosphorylation sites in Sap-1a

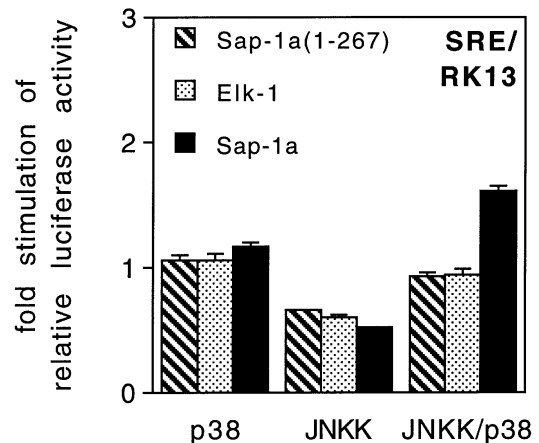
Sap-1a contains >10 potential MAPK phosphorylation sites (S/TP) within its C-terminal transactivation domain. As a first step in identifying serine/threonine residues being phosphorylated by p38 MAPK, GST-Sap was phosphorylated *in vitro* with either activated ERK-1 or p38 MAPK in the presence of [ $\gamma$ - $^{32}$ P]ATP. Purified phosphorylated GST-Sap was then cleaved with the protease chymotrypsin and the resulting phosphopeptides were resolved on cellulose thin layer plates in the first dimension by electrophoresis and in the second dimension by ascending chromatography. A complex pattern of phospho-



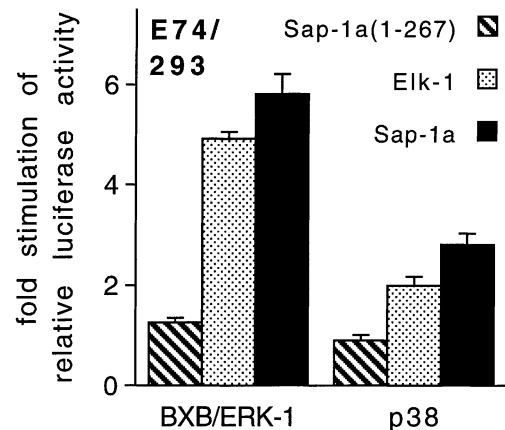
**Fig. 3.** Effect of the inhibitor SB202190 and upstream p38 MAPK activators on Sap-1a-mediated transactivation. **(A)** p38 MAPK (75 ng vector), JNKK or BXB and ERK-1 were co-transfected with Sap-1a and a *c-fos* SRE-driven luciferase reporter into 293 cells. Where indicated, 5  $\mu$ M SB202190 was administered to the culture medium 24 and 12 h prior to cell harvest. **(B)** TAK1 potentiates stimulation of Sap-1a- but not Elk-1-mediated *c-fos* SRE-dependent transcription by p38 MAPK (25 ng vector) in 293 cells.

peptides was observable (Figure 6A), but the patterns of phosphorylation by ERK-1 and p38 MAPK were nearly identical (the d' and e' phosphopeptides were also faintly observable in the original autoradiogram of Sap-1a phosphorylated by p38 MAPK).

Mutation of the potential MAPK phosphorylation sites Ser381 and Ser387 to alanine, which have been shown to be important sites in the regulation of Sap-1a by ERKs (Janknecht *et al.*, 1995b; Price *et al.*, 1995), revealed that phosphopeptides 1'-5' were due to phosphorylation at these sites. Notably, Ser381 and Ser387 were targeted by both ERK-1 and p38 MAPK to a similar degree (compare peptides 1'-5' with peptides a'-c'). Next, we analyzed the pattern of *in vivo* phosphorylation of GAL4-Sap, which was metabolically labeled with  $^{32}$ P<sub>i</sub> in 293 cells expressing either p38 MAPK or the combination of



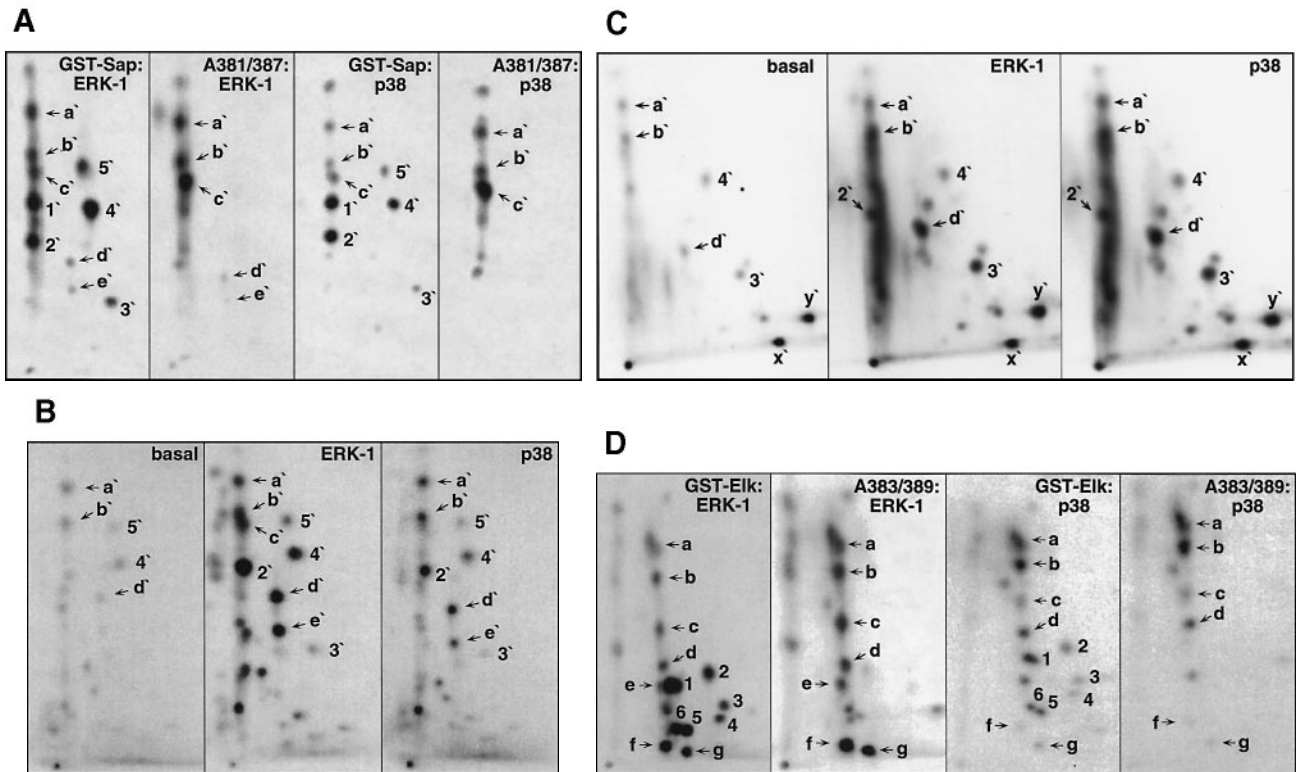
**Fig. 4.** Inefficient activation of Sap-1a-mediated *c-fos* SRE-dependent transcription by p38 MAPK (30 ng vector) in RK13 cells. Where indicated, a JNKK expression vector was co-transfected.



**Fig. 5.** Activation of an E74 binding site-driven luciferase reporter gene by indicated TCFs upon co-transfection of either BXB and ERK-1 or p38 MAPK in 293 cells.

ERK-1 and BXB. Again, phosphorylation of Sap-1a in the presence of ERK-1 and p38 MAPK occurred at the same sites *in vivo* (Figure 6B) and the phosphopeptide pattern obtained from *in vivo* labeled Sap-1a matched that seen upon *in vitro* phosphorylation fairly well. However, peptide 1' was not observable and peptides d' and e', which vanished upon mutating both Thr420 and Ser425 to alanine (data not shown), were much more prominent. In conclusion, Ser381 and Ser387 in Sap-1a are prominent phosphorylation sites for p38 MAPK both *in vitro* and *in vivo*.

In order to verify that the identified phosphorylation sites were also targeted by MAPKs in full-length Sap-1a *in vivo*, Myc epitope-tagged full-length Sap-1a was transiently expressed in 293 cells either alone or with BXB and ERK-1 or p38 MAPK and phosphopeptide analysis was performed after *in vivo* labeling with  $^{32}$ P<sub>i</sub> (Figure 6C). As with GAL4-Sap, phosphorylation of Myc-Sap-1a was induced by both BXB and ERK-1 and p38 MAPK *in vivo*. Specifically, increased labeling of phosphopeptides 2'-4', which indicates phosphorylation at Ser381 and Ser387, as well as phosphopeptide d', which indicates phosphorylation at residues 420 and 425, was observed. However, the phosphopeptide pattern of Myc-Sap-1a displayed some differences to GAL4-Sap, which

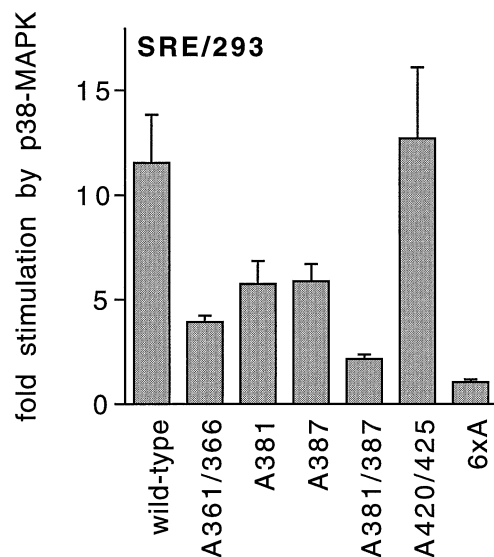


**Fig. 6.** Phosphopeptide analyses. **(A)** Two-dimensional analysis of chymotryptic phosphopeptides derived from GST-Sap or the respective double mutant Ala381/Ala387 after *in vitro* phosphorylation with p38 MAPK or ERK-1. Electrophoresis was used for the first dimension (anode on the left) and ascending chromatography for the second. Chymotryptic phosphopeptide analysis of **(B)** GAL4-Sap or **(C)** Myc-Sap-1a derived from  $^{32}\text{P}_i$ -labeled 293 cells. Where indicated, ERK-1 with its upstream activator BXB or p38 MAPK were co-expressed. **(D)** Chymotryptic phosphopeptides derived from GST-Elk or the respective double mutant Ala383/Ala389 after *in vitro* phosphorylation with ERK-1 or p38 MAPK.

may be due to additional MAPK phosphorylation sites outside the transactivation domain. In this connection, phosphorylation of the unidentified peptides x' and y', which were not observed in GAL4-Sap, appears to be nearly unaltered by BXB and ERK-1 or p38 MAPK expression, which might reflect a constitutive phosphorylation of Sap-1a by another protein kinase. Nevertheless, we conclude that Ser381 and Ser387 especially are also targeted in full-length Sap-1a by both ERK-1 and p38-MAPK *in vivo*.

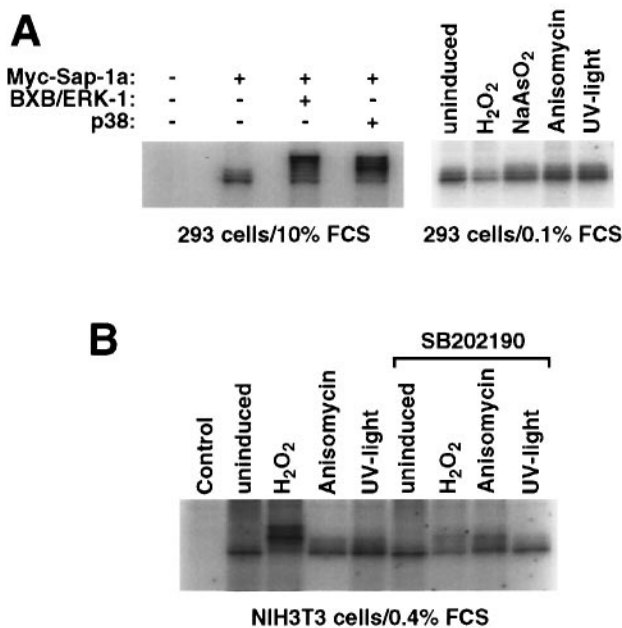
Phosphopeptide analysis of Elk-1 indicated that phosphorylation at Ser383 and Ser389 (peptides 1–6), which are homologous to Ser381 and Ser387 in Sap-1a, as well as within the unidentified peptides f and g was much less efficiently catalyzed by p38 MAPK than by ERK-1 *in vitro* (compare with peptides a–d in Figure 6D). Previous studies showed that Ser383 and Ser389 were phosphorylated *in vivo* and that blocking phosphorylation at these sites abolished the ability of Elk-1 to transactivate (Janknecht *et al.*, 1993; Marais *et al.*, 1993; Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Whitmarsh *et al.*, 1995). Thus, inefficient phosphorylation of Ser383 and Ser389 by p38 MAPK is most likely the reason why Elk-1-mediated transcription is only poorly stimulated by p38 MAPK.

Mutation of either Ser381 or Ser387 to alanine in Sap-1a reduced its transactivation potential upon p38 MAPK phosphorylation by half, while the double alanine mutant Ala381/Ala387 was >5-fold less active than the wild-type (Figure 7). Also, mutation of the phosphorylation sites



**Fig. 7.** Mutation of p38 MAPK sites in Sap-1a to alanine affects *c-fos* SRE-dependent transcription upon co-transfection of p38 MAPK in 293 cells. The 6×A mutant of Sap-1a has alanines at positions 361, 366, 381, 387, 420 and 425.

Thr361 and Thr366 to alanine reduced the transactivation potential of Sap-1a by ~3-fold, while alanines at residues 420 and 425 had no effect. Joint mutation of all six aforementioned phosphorylation sites (6×A) blocked activation by p38 MAPK completely. These data indicate that phosphorylation of Sap-1a, especially at Ser381 and



**Fig. 8.** Phosphorylation of Sap-1a upon stimulation of cells. (A) The left panel shows an autoradiogram after SDS-PAGE of Myc-Sap-1a immunoprecipitated from 293 cells which were continuously growing in medium supplemented with 10% fetal calf serum (FCS) and labeled with  $^{32}\text{P}_i$ . Where indicated, BXB and ERK-1 or p38 MAPK were co-expressed. The right panel depicts phosphorylated Myc-Sap-1a immunoprecipitated from serum starved,  $^{32}\text{P}_i$ -labeled 293 cells which had been stimulated for 30 min with 2 mM  $\text{H}_2\text{O}_2$ , 0.25 mM  $\text{NaAsO}_2$ , 10  $\mu\text{g}/\text{ml}$  anisomycin or UV light (20 s at 8  $\text{W}/\text{m}^2$ ). (B) Similarly, transiently expressed Myc-Sap-1a was immunoprecipitated from serum starved,  $^{32}\text{P}_i$ -labeled NIH 3T3 cells induced for 30 min with 2 mM  $\text{H}_2\text{O}_2$ , 10  $\mu\text{g}/\text{ml}$  anisomycin or UV light (20 s at 8  $\text{W}/\text{m}^2$ ). Where indicated, 5  $\mu\text{M}$  SB202190 was administered to the cells 1 h prior to induction. Control denotes NIH 3T3 cells which were not transfected with Myc-Sap-1a.

Ser387, by p38 MAPK is indeed required for p38 MAPK to activate *c-fos* SRE-dependent transcription.

#### Induction of phosphorylation of Sap-1a by extracellular stimuli

Since our studies so far relied on overexpression of p38 MAPK, we wanted to confirm that Sap-1a phosphorylation could also be induced by extracellular stimuli via endogenous p38 MAPK. Such phosphorylation of Sap-1a can be detected by a decrease in mobility on an SDS-polyacrylamide gel, as observable after activation of Myc-Sap-1a in 293 cells by BXB and ERK-1 or p38 MAPK overexpression *in vivo* (Figure 8A, left panel). However, when 293 cells were serum-starved for >18 h and then stimulated with known p38 MAPK inducers, such as  $\text{H}_2\text{O}_2$ , sodium arsenite ( $\text{NaAsO}_2$ ), anisomycin or UV light, we were unable to detect an increase in Myc-Sap-1a phosphorylation (Figure 8A, right panel). This might be due to the fact that 293 cells are highly transformed and thus do not become quiescent upon serum starvation.

Therefore, we also analyzed NIH 3T3 cells that were serum deprived and then stimulated with either  $\text{H}_2\text{O}_2$ , anisomycin or UV light. All of these stimuli resulted in increased phosphorylation of Myc-Sap-1a, most prominently seen with  $\text{H}_2\text{O}_2$  (Figure 8B). We next addressed the question whether this phosphorylation could be blocked by administration of the p38 MAPK inhibitor SB202190.

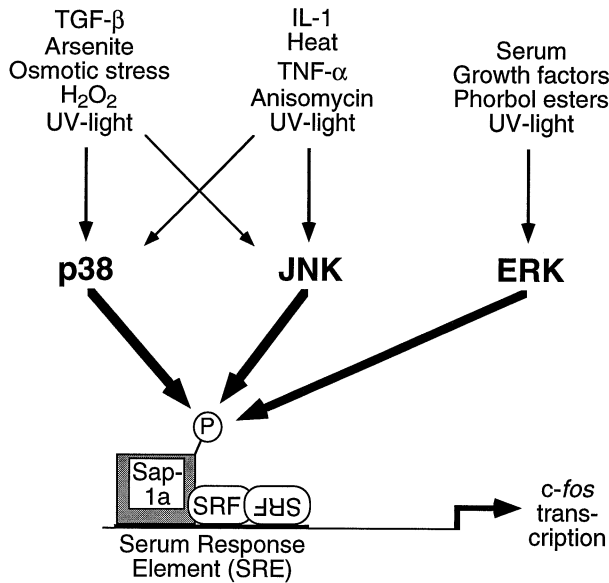
Phosphorylation of Myc-Sap-1a induced by anisomycin or UV light was unaffected by SB202190 (Figure 8B). This could be because p38 MAPK is not activated by anisomycin and UV light in NIH 3T3 cells. Alternatively, the JNKs, which can also be activated by anisomycin and UV light, completely compensate for inhibition of p38 MAPK, consistent with our results that Sap-1a is phosphorylated and activated by JNKs (Janknecht and Hunter, 1997). However, Myc-Sap-1a was overall in a less phosphorylated state upon stimulation with  $\text{H}_2\text{O}_2$  in the presence of SB202190. Thus, a part of the phosphorylation of Sap-1a upon stimulation with  $\text{H}_2\text{O}_2$  is due to p38 MAPK, while the rest can most likely be attributed to JNKs that are also inducible by  $\text{H}_2\text{O}_2$ . Altogether, these data indicate that Sap-1a can be phosphorylated *in vivo* by endogenous p38 MAPK and additionally by JNKs.

#### Discussion

In this report we have demonstrated that the ETS family transcription factor Sap-1a is phosphorylated and thus activated by p38 MAPK by several criteria: (i) p38 MAPK efficiently activates *c-fos* SRE-dependent transcription in 293 cells solely in the presence of Sap-1a; (ii) Sap-1a is phosphorylated by p38 MAPK *in vitro*; (iii) phosphorylation of Sap-1a by p38 MAPK *in vitro* occurs at the same sites in the transactivation domain as phosphorylation of Sap-1a *in vivo* upon p38 MAPK expression; (iv) mutation of the prominent p38 MAPK sites Ser381 and Ser387 in Sap-1a drastically reduces the ability of p38 MAPK to activate Sap-1a-mediated *c-fos* SRE stimulation. Thus, we have identified a novel effector of p38 MAPK and Sap-1a may be critical for the nuclear response elicited by signals funneling through p38 MAPK.

All three hitherto identified TCFs (Elk-1, Sap-1a and Sap-2/Erp/Net) are activated by ERKs and are thus capable of inducing *c-fos* gene transcription (Janknecht *et al.*, 1993, 1995b; Marais *et al.*, 1993; Giovane *et al.*, 1994; Price *et al.*, 1995). Also, Elk-1 (Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Whitmarsh *et al.*, 1995) and Sap-1a (Janknecht and Hunter, 1997) are activated by the JNK subclass of MAPKs and can thus perform similar functions downstream of JNKs, the latter in apparent contradiction of two recent reports (Whitmarsh *et al.*, 1995; Strahl *et al.*, 1996). However, Whitmarsh and colleagues conclusion was based solely on *in vitro* phosphorylation experiments utilizing the activation domain of Sap-1a as a substrate, which showed that Sap-1a is not efficiently phosphorylated by JNKs *in vitro*; they did not analyze full-length Sap-1a *in vivo*. Strahl and colleagues could not detect phosphorylation of Sap-1a upon anisomycin treatment of NIH 3T3 cells, but did not investigate Sap-1a phosphorylation upon stimulation of NIH 3T3 cells with other known JNK inducers. Since we observed only a modest increase in Sap-1a phosphorylation after anisomycin stimulation of NIH 3T3 cells, this difference might be explained by the facts that Strahl *et al.* (1996) employed a 100-fold lower concentration of anisomycin, performed a 10 min shorter induction and most likely utilized a different strain of NIH 3T3 cells.

In this study we have demonstrated that Elk-1 is only poorly activated by p38 MAPK, since important phosphorylation sites, especially Ser383 and Ser389, in



**Fig. 9.** Convergence of different signaling pathways on Sap-1a at the *c-fos* promoter.

Elk-1 are not as efficiently phosphorylated by p38 MAPK as they are by ERK-1. Therefore, mitogens such as growth factors or phorbol esters, which preferentially stimulate ERKs, as well as heat, the cytokine IL-1 or the apoptosis inducer TNF- $\alpha$ , which mainly activate JNKs (Cahill *et al.*, 1996; Verheij *et al.*, 1996), can mediate *c-fos* gene transcription via both Elk-1 and Sap-1a, but only Sap-1a serves as a convergence point for all three major classes of mammalian MAPKs (Figure 9) and thus is also stimulated by signals such as TGF- $\beta$  or osmotic shock that preferentially induce p38 MAPK activity (Yamaguchi *et al.*, 1995; Cahill *et al.*, 1996). Since TCFs are expressed in a tissue-specific manner (Giovane *et al.*, 1994), the ratio of Sap-1a to Elk-1 will vary in different cell types and may thus dictate whether *c-fos* can be efficiently activated by p38 MAPK, since both proteins compete for binding to the SRE.

In RK13 cells, which, like 293 cells, originate from kidney epithelium, Sap-1a was not responsive to p38 MAPK activated by JNKK. We do not think that this is due to a large surplus of endogenous Elk-1 in RK13 cells competitively inhibiting Sap-1a, since exogenous Sap-1a was expressed at such levels as to replace any endogenous TCFs from a *c-fos* SRE within the reporter construct (Janknecht *et al.*, 1995b). Furthermore, Sap-1a is in principle activatable in RK13 cells by both ERKs and JNKs (Janknecht *et al.*, 1995b; Janknecht and Hunter, 1997), suggesting that it is not due to Sap-1a that p38 MAPK cannot activate Sap-1a-mediated *c-fos* transcription in RK13 cells. Rather, an inhibitor of p38 MAPK catalytic activity, such as the cell cycle inhibitor p21<sup>WAF1/CIP1</sup> (Shim *et al.*, 1996) could be responsible. If, for instance, RK13 cells have a high load of p21<sup>WAF1/CIP1</sup> in comparison with 293 cells or, alternatively, a low amount of proteins capable of sequestering p21<sup>WAF1/CIP1</sup>, signal transduction could be blocked at the level of p38 MAPK. This explanation would be compatible with the fact that p38 MAPK, when co-expressed with JNKK in RK13 cells, is active *in vitro* after immunoprecipitation, since the putative inhibitor would have been washed away during this

procedure. In conclusion, our results point to the fact that p38 MAPK may be activated but cannot function and that thus *c-fos* induction may be prevented in certain cell types.

Furthermore, our data indicate that Sap-1a is much more active in conjunction with the SRF protein at the *c-fos* SRE than at the ETS binding site E74 in 293 cells upon phosphorylation by ERK-1 as well as p38 MAPK. This suggests that Sap-1a is truly a TCF in 293 cells and is almost unable to stimulate genes solely regulated by an ETS binding site. However, we have shown previously that Sap-1a acts with comparable strength at the *c-fos* SRE and the E74 site in RK13 cells after ERK-1 stimulation (Janknecht *et al.*, 1995b). How can such a difference be explained? Sap-1a interacts with the SRF protein at the *c-fos* SRE and this may result in an altered protein conformation in comparison with the situation at the E74 site. This in turn may cause recruitment of different coactivators, one of which may be CBP (Janknecht and Hunter, 1996; Janknecht and Nordheim, 1996). In this scenario, 293 cells may have lower levels of the coactivator required for Sap-1a-mediated transactivation via the E74 site as compared with RK13 cells. Thus, activation of a transcription factor such as Sap-1a by phosphorylation may not result in stimulation of gene transcription because the relay of signals stops at the junction between the transcription factor and the basal transcription machinery within certain promoter contexts. This could be one reason why different cells respond differently to extracellular stimuli.

In conclusion, our study implies that activation of certain gene promoters in response to extracellular stimuli can be regulated at several levels within a signaling pathway. For the TCFs, productive signaling requires not only activation of an appropriate MAPK, but also phosphorylation of the ETS family protein at the right sites and the correct constellation of accessory components to make functional contacts between the phosphorylated TCF and the transcription initiation complex and thereby trigger RNA polymerase II. Thus, although extracellular signals may initiate an intracellular signaling cascade, the transmission of information can stop at different levels of this cascade, which may safeguard the cell type-specific gene activation required for the development and homeostasis of a complex organism.

## Materials and methods

### Transient transfection and reporter gene assay

Cells were grown to 25% confluence on 6 cm dishes and then transfected by the calcium phosphate co-precipitation method with the indicated plasmids as described previously (Janknecht *et al.*, 1993). Cells were harvested 36 h after transfection and luciferase activity was determined utilizing D-luciferin as substrate (Janknecht *et al.*, 1995b). Typically, 2  $\mu$ g luciferase reporter plasmid, 50 ng TCF expression vector and 2 (JNKK), 0.5 (BXB), 1 (ERK-1) or 0.5  $\mu$ g (TAK1) protein kinase expression vector were employed. Normalization of transfection efficiency was done using a co-transfected  $\beta$ -galactosidase expression vector. Fold activation of luciferase activity was calculated relative to cells transfected without any kinase expression vector.

### Production of GST fusion proteins

GST fusion proteins were expressed in *Escherichia coli* BL21. Enrichment of the fusion proteins was on Ni<sup>2+</sup>-NTA-agarose columns (Qiagen) in 6 M guanidine HCl by virtue of a histidine tag present at the junction between the GST moiety and the fused TCF C-terminal transactivation domain. Proteins were renatured by dialysis against 25 mM HEPES,

pH 7.6, 100 mM NaCl, 5 mM dithiothreitol (DTT), 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF).

#### Gel retardation assay and in vitro phosphorylation

Gel retardation assays were performed with extracts from transiently transfected 293 cells with either a <sup>32</sup>P-labeled E74 oligonucleotide or with SRF protein plus <sup>32</sup>P-labeled *c-fos* SRE. Complexes were resolved on a 4.5% native polyacrylamide gel (Janknecht *et al.*, 1995b). *In vitro* phosphorylation was performed with flag-tagged p38 MAPK immunoprecipitated with the M2 antibody (IBI) or with HA-tagged ERK-1 co-transfected with constitutively activated Raf-1 (BXB) and immunoprecipitated with the 12CA5 antibody (Boehringer Mannheim) (Janknecht *et al.*, 1993).

#### In vivo phosphorylation and phosphopeptide mapping

293 cells were transiently transfected with the G/S268-431 expression vector (Janknecht *et al.*, 1995b), which encodes the GAL4 DNA binding domain fused to the C-terminal activation domain of Sap-1a, or, alternatively, with an N-terminally 6×Myc epitope-tagged full-length Sap-1a expression vector and either p38 MAPK or ERK-1 and BXB expression vectors. Thirty-six hours post-transfection cells were metabolically labeled with <sup>32</sup>P<sub>i</sub> for 5 h. Then, the GAL4-Sap fusion protein or Myc-Sap-1a was immunoprecipitated with anti-GAL4 antibodies or anti-Myc tag antibodies (Santa Cruz Biotechnology) respectively, the immunoprecipitates boiled in Laemmli sample buffer and subjected to SDS-PAGE. The phosphorylated GAL4-Sap fusion protein or Myc-Sap-1a was eluted from the gel, digested with chymotrypsin and the resulting phosphopeptides resolved by electrophoresis in the first dimension (pH 1.9 buffer, 1.3 kV, 25 min) and by ascending chromatography employing phosphochromatography buffer in the second dimension on cellulose thin layer plates (van der Geer *et al.*, 1994).

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